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The N-terminal region of cystatin A (stefin A) binds to papain subsequent to the two hairpin loops of the inhibitor. Demonstration of two-step binding by rapid-kinetic studies of cystatin A labeled at the N-terminus with a fluorescent reporter group

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Abstract

The three-dimensional structures of cystatins, and other evidence, suggest that the flexible N-terminal region of these inhibitors may bind to target proteinases independent of the two rigid hairpin loops forming the remainder of the inhibitory surface. In an attempt to demonstrate such two-step binding, which could not be identified in previous kinetics studies, we introduced a cysteine residue before the N-terminus of cystatin A and labeled this residue with fluorescent probes. Binding of AANS- and AEDANS-labeled cystatin A to papain resulted in ~4-fold and 1.2-fold increases of probe fluorescence, respectively, reflecting the interaction of the N-terminal region with the enzyme. Observed pseudo-first-order rate constants, measured by the loss of papain activity in the presence of a fluorogenic substrate, for the reaction of the enzyme with excess AANS-cystatin A increased linearly with the concentration of the latter. In contrast, pseudo-first-order rate constants, obtained from measurements of the change of probe fluorescence with either excess enzyme or labeled inhibitor, showed an identical hyperbolic dependence on the concentration of the reactant in excess. This dependence demonstrates that the binding occurs in two steps, and implies that the labeled N-terminal region of cystatin A interacts with the proteinase in the second step, subsequent to the hairpin loops. The comparable affinities and dissociation rate constants for the binding of labeled and unlabeled cystatin A to papain indicate that the label did not appreciably perturb the interaction, and that unlabeled cystatin therefore also binds in a similar two-step manner. Such independent binding of the N-terminal regions of cystatins to target proteinases after the hairpin loops may be characteristic of most cystatin–proteinase reactions.

Keywords: cystatin; fluorescence labeling; N-terminal region; papain; stefin; stopped-flow fluorescence; two-step binding

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Abbreviations: AANS, (2-(4′-acetamido)anilino)naphthalene-6-sulfonic acid; AANS–cystatin A, cystatin A labeled with an AANS group at a Cys residue introduced in the N-terminus; AEDANS, 5-((((2-acetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; AEDANS–cystatin A, cystatin A labeled with an AEDANS group introduced in the N-terminus; app, subscript denoting an apparent equilibrium or rate constant measured in the presence of an enzyme substrate; +Cys-cystatin A, a variant of cystatin A with an extra Cys residue introduced before Met-1 of the wild-type inhibitor; DTT, dithiothreitol; His-tag, 10 consecutive histidine residues fused to an expressed protein; IAANS, (2-(4′-iodoacetamido)anilino)naphthalene-6-sulfonic acid; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; k_{ass}, bimolecular association rate constant; K_d, dissociation equilibrium constant; k_{diss}, dissociation rate constant; k_{obs}, observed pseudo-first-order rate constant; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid.

Cystatin A (also called stefin A) is a 98-amino acid protein belonging to family I, or stefin family, of the cystatin superfamily of cysteine proteinase inhibitors. Cystatins efficiently inactivate papain-like proteinases from widely different sources, for example, lysosomal enzymes, like cathepsins B, H, K, L, and S, plant enzymes, like papain and actinidin, and proteinases from pathogens, like cruzipain (for reviews, see Barrett et al., 1986; Turk & Bode, 1991; Turk et al., 1997). The target proteinases are inhibited as a result of the cystatins trapping them in tight, although reversible, equimolar complexes, in which the active site cleft of the enzyme is blocked. The X-ray structure of chicken cystatin, the first structure of a cystatin to be solved (Bode et al., 1988), showed that evolutionarily conserved residues from the N-terminal region and two hairpin loops, one central and one C-terminal, are exposed on the surface of the protein, forming a wedge-shaped edge. In computer docking experiments, this wedge could fit into the active site cleft of papain without major conformational changes of either of the molecules (Bode et al., 1988), thereby accounting for the formation of a tight complex. The crystal structure of a complex between papain and cystatin B (stefin B) resolved later (Stubbs et al., 1990) essentially verified this mode of interaction. Further support for the reactions of cystatins with papain, and other proteinases having easily accessible active site clefts, occurring with minimal conformational changes of the two proteins has come from analyses of the kinetics of binding of the inhibitors to such enzymes. These kinetics, monitored by changes of tryptophan fluorescence, are thus consistent with a rapid, almost diffusion-controlled, one-step binding reaction (Björk et al., 1989; Björk & Ylinenjärvi, 1990; Lindahl et al., 1992a; Turk et al., 1992, 1995; Pol et al., 1995). In contrast, cystatin C has been shown to bind to cathepsin B, an enzyme in which the active site cleft is partially covered by the "occluding loop" (Musil et al., 1991), by a two-step mechanism, presumably because this loop has to be displaced in a second step before a tight complex can be formed (Nycander et al., 1998).

Studies of the solution structures of cystatin A (stefin A) (Martin et al., 1995), chicken cystatin (Dieckmann et al., 1993), and cystatin C (Ekiel et al., 1997) by NMR have revealed that the N-terminal portion of the inhibitory wedge is highly flexible, in contrast with the more rigid first and second binding loops. Truncation of the N-terminal region substantially increases the rate of dissociation of complexes of cystatins with proteinases having accessible active sites, such as papain, consistent with this region participating in anchoring the inhibitor to the proteinase (Lindahl et al., 1992b; Björk et al., 1994; Estrada et al., 1999). However, such truncation does not affect the rate of complex formation with these proteinases, indicating that the N-terminal region is not necessary for the fast association of the two molecules (Lindahl et al., 1992b; Björk et al., 1994; Estrada et al., 1999). The N-terminal region may thus bind to proteinases with accessible active sites subsequent to the other binding regions in a reaction that was not detected by the kinetics studies. However, interaction of the N-terminal region with cathepsin B appears to precede and facilitate the displacement of the occluding loop in the binding of cystatins to this enzyme (Björk et al., 1994, 1995, 1996; Nycander et al., 1998).

In this work, we have used the efficient expression system developed for the production of human cystatin A (Pol et al., 1995; Estrada et al., 1998) to express a variant of the inhibitor with an extra cysteine residue before the N-terminal methionine residue of the wild-type protein. Fluorescent probes, AANS or AEDANS, were attached to this cysteine as specific reporter groups of the interactions involving the N-terminal region of the inhibitor in the binding to papain. The results do indeed indicate that the N-terminal region binds to papain in a second step after the remainder of the inhibitory wedge has interacted with the enzyme.

Results

Expression and labeling

The +Cys-cystatin A variant, having an extra Cys at the N-terminus, was expressed with a His-tag, and purified essentially as in previous work (Pol et al., 1995; Estrada et al., 1998), although with minor modifications due to the presence of the free cysteine residue. As the formation of dimers, like those seen with cystatin B (Turk et al., 1992), might have interfered with the cleavage of the His-tag by enterokinase, the protein was treated with DTT before incubation with the enzyme. About 70% of the His-tagged protein was recovered as cleaved inhibitor, giving a yield of ~5 mg/L of induced culture. The purified variant gave only one band in SDS-PAGE, and N-terminal sequence analysis confirmed the presence of a cysteine preceding Met-1 of wild-type cystatin A. Labeling of the cysteine residue with the fluorescent reporter groups, AANS or AEDANS, resulted in incorporation of 1.05 and 1.1 mol of label per mol of protein, respectively, as determined from the absorbance of the label and the protein concentration, measured by absorbance at 280 nm. Because of the large contribution of the AANS group to the latter absorbance, the protein concentration of AANS–cystatin A was also measured by quantitative amino acid analysis, which gave an incorporation of 1.1 mol of label per mole of protein. Both the AANS- and AEDANS-labeled cystatin A forms bound to papain with stoichiometries of 0.95–1.05, as shown by titrations monitored by either the increase of probe fluorescence accompanying the binding (see below) or by the loss of enzyme activity.

Fluorescence changes on binding to papain

Fluorescence spectra showed that the binding of AANS–cystatin A to papain was accompanied by an appreciable ~4.2-fold increase of probe fluorescence and a shift in the wavelength of the emission maximum from 462 to 453 nm. The fluorescence change on binding of AEDANS–cystatin A to papain was much smaller, ~1.2-fold, with a wavelength shift from 493 to 489 nm.

Kinetics of AANS–cystatin A binding to papain

The observed pseudo-first-order rate constant k_{obs} for reactions of AANS–cystatin A with papain, measured by monitoring changes of probe fluorescence in a stopped-flow instrument, showed a hyperbolic dependence on the concentration of the reactant in excess (Fig. 1A). This dependence was similar for experiments done with either an excess of enzyme or labeled inhibitor. The data were fitted to the hyperbolic function $k_{obs} = k_{lim}[R]_0/(K_{0.5} + [R]_0)$, in which k_{lim} represents the limiting rate constant for the reaction, $K_{0.5}$ is the concentration of the reactant in excess at which half of the limiting rate constant in reached, and $[R]_0$ is the concentration of the reactant in excess. Both sets of data gave good fits to this equation, with similar values for k_{lim} of $230 \pm 7\ s^{-1}$ and $250 \pm 50\ s^{-1}$ and for $K_{0.5}$ of $(9.8 \pm 0.6) \times 10^{-6}$ M and $(11 \pm 3) \times 10^{-6}$ M for analyses with excess of papain and inhibitor, respectively (Fig. 1A).

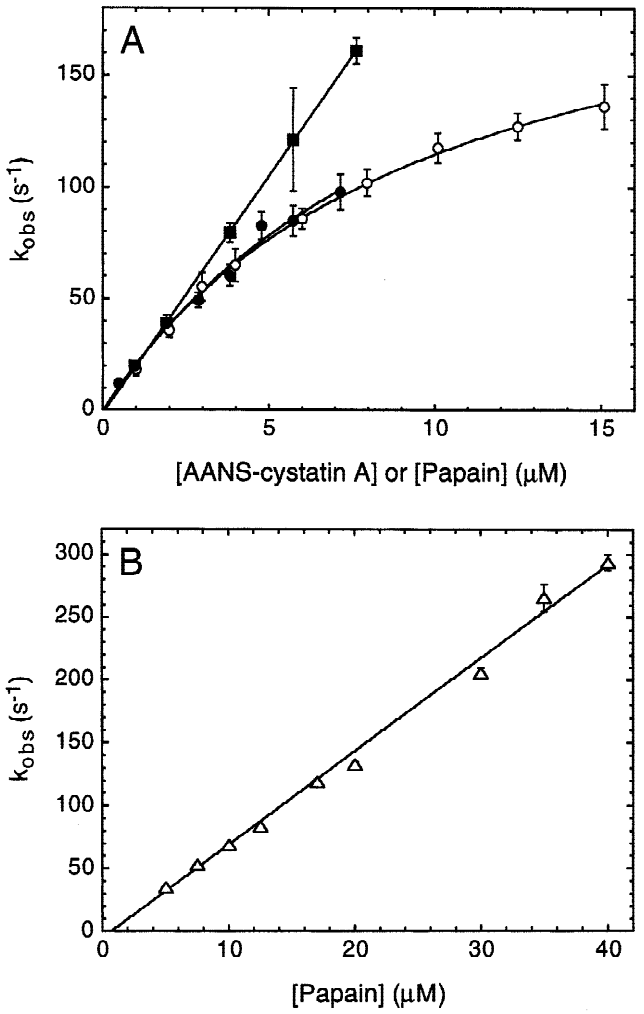

Fig. 1. Pseudo-first-order rate constants k_{obs} for the binding of AANS- or AEDANS–cystatin A to papain as a function of the concentration of the reactant in excess. **A:** AANS–cystatin A. (■) Dependence of k_{obs}, monitored by the loss of enzyme activity, on AANS–cystatin A concentration; (●) dependence of k_{obs}, monitored by the increase of AANS fluorescence, on AANS–cystatin A concentration; (○) dependence of k_{obs}, monitored by the increase of AANS fluorescence, on papain concentration. **B:** AEDANS–cystatin A. (△) dependence of k_{obs}, monitored by the increase of AEDANS fluorescence, on papain concentration. Values of k_{obs} monitored by the loss of enzyme activity were corrected for substrate competition. The vertical bars represent the standard errors. Error bars not shown lie within the dimensions of the symbols. The solid lines represent linear or nonlinear regression fits.

In contrast to this behavior, k_{obs} for the inhibition of papain by an excess of AANS–cystatin A, derived from progress curves monitored by substrate cleavage in the stopped-flow fluorimeter and corrected for substrate competition, increased linearly with inhibitor concentration up to the highest concentration that could be analyzed (Fig. 1A). The second-order association rate constant k_{ass} obtained from the slope of this plot agreed well with the k_{ass}-values calculated from the initial slopes of the fits of k_{obs} monitored by probe fluorescence (Table 1). These values are about sevenfold higher than that for the reaction of the unlabeled wild-type inhibitor with papain (Table 1).

The intercept on the ordinate in the kinetic analysis of enzyme inhibition in the presence of substrate, which reflects the overall rate constant for the dissociation of the AANS–cystatin A–papain complex k_{diss} was indistinguishable from zero. This rate constant was therefore determined in separate experiments by a displacement procedure and was identical, within experimental error, to the value for the unlabeled wild-type inhibitor (Table 1). The overall dissociation equilibrium constant K_d of the complex was calculated from k_{diss} and k_{ass} (Table 1), and was eightfold lower than that of the complex of papain with unlabeled cystatin A, due almost exclusively to the higher k_{ass}.

Kinetics of AEDANS–cystatin A binding to papain

k_{obs} for reactions of AEDANS–cystatin A with papain, measured by monitoring the change of probe fluorescence with excess enzyme, increased linearly with papain concentration up to as high concentration as could be studied (Fig. 1B). Analyses with an excess of labeled inhibitor were not possible because of the low fluorescence change. The value of k_{ass} obtained from this plot was in good agreement with that measured by monitoring enzyme inhibition in the presence of substrate with excess inhibitor in a lower concentration range (Table 1). The two k_{ass} values were about twofold higher than that for unlabeled wild-type cystatin A (Table 1). A k_{diss} about half of that of the unlabeled inhibitor was measured by a displacement assay, which together with the higher k_{ass} led to about a fourfold lower K_d (Table 1).

Discussion

The fluorescence changes observed on binding to papain of cystatin A with either of the two fluorescent probes, AANS or AEDANS, attached to the N-terminus are consistent with transfer of the probes to a more hydrophobic environment. These changes must reflect the interaction of the N-terminal region with the proteinase shown previously to stabilize cystatin–proteinase complexes (Machleidt et al., 1989; Abrahamson et al., 1991; Auerswald et al., 1994; Björk et al., 1994; Hall et al., 1995; Estrada et al., 1998, 1999). AANS was found to be an appreciably more sensitive reporter group of this interaction than AEDANS.

The same hyperbolic concentration dependence of k_{obs} for the binding of AANS–cystatin A to papain, monitored by changes of probe fluorescence, was seen with excess enzyme or labeled inhibitor. This behavior indicates that the binding occurs in two steps (Fersht, 1985):

$$\mathrm{P} + \mathrm{I} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{PI} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} \mathrm{PI}^*.$$

In the first step, proteinase (P) and inhibitor (I) form an initial complex (PI), which is converted in the second step to the final complex (PI*). The observed hyperbolic dependence requires that the fluorescence change is induced in the second step, which therefore must involve the interaction of the labeled N-terminal region with the proteinase. Consequently, the first step must reflect an initial binding of the remainder of the binding region, i.e., the two hairpin loops, to the enzyme. The hyperbolic concentration dependence also implies that the binding of the AANS-labeled N-terminal region is sufficiently slow to be seen as a separate step in the stopped-flow time frame. As discussed in detail later, the linear dependence seen with AEDANS–cystatin A suggests that the bind-

Table 1. *Association rate constants (k_{ass}), dissociation rate constants (k_{diss}), and dissociation equilibrium constants (K_d) for the interactions of wild-type cystatin A, AANS–cystatin A, or AEDANS-cystatin A with papain*[a]

Cystatin A form	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_d (M)
Wild-type	3.1×10^6 [1]	5.5×10^{-7} [1]	1.8×10^{-13} [1]
AANS	$(2.1 \pm 0.01) \times 10^7$ (5)[b] [7]	$(5.3 \pm 0.8) \times 10^{-7}$ (2) [1]	2.3×10^{-14}[c] [8]
	$(2.3 \pm 0.07) \times 10^7$ (9)[d] [7]		
	$(2.3 \pm 0.2) \times 10^7$ (8)[e] [7]		
AEDANS	$(6.1 \pm 0.1) \times 10^6$ (9)[b] [2]	$(2.7 \pm 0.1) \times 10^{-7}$ (2) [2]	4.4×10^{-14}[c] [4]
	$(7.5 \pm 0.2) \times 10^6$ (9)[d] [2]		

[a]The values for wild-type cystatin A are taken from previous work (Pol et al., 1995) and are shown for comparison. Measured values are given with standard error or range (if derived from only two measurements) and with the number of measurements in parentheses. Calculated values and values obtained previously are given without errors. Relative values, defined as $k_{ass,\,labeled}/k_{ass,\,wild\text{-}type}$, $k_{diss,\,wild\text{-}type}/k_{diss,\,labeled}$, and $K_{d,\,wild\text{-}type}/K_{d,\,labeled}$, are given within square brackets. Relative values >1 thus reflect changes of k_{ass}, k_{diss}, and K_d expected to result in increased binding affinity.

[b]From measurements of papain inhibition by excess labeled cystatin in the presence of substrate.

[c]Calculated from k_{ass} and k_{diss}.

[d]From measurements of the change of probe fluorescence with excess papain. Calculated from the initial slope ($= k_{lim}/K_{0.5}$) of the plot in Figure 1A for AANS–cystatin A and from the slope of the plot in Figure 1B for AEDANS–cystatin A.

[e]From measurements of the change of probe fluorescence with excess labeled cystatin. Calculated from the initial slope of the plot in Figure 1A.

ing of the N-terminal region labeled with this probe is too rapid to be distinguished as a separate step.

The use of two different reporters of the AANS–cystatin A–papain reaction, inactivation of the enzyme and binding of the N-terminal end, enables individual rate constants for the two-step reaction to be estimated. The limiting rate constant k_{lim} of the hyperbolic concentration dependence must represent the forward rate constant k_{+2} of the second step involving binding of the labeled N-terminal region, which is rate-limiting at high reactant concentrations. k_{+2} should thus be $\sim 230\ s^{-1}$. Moreover, for the first step, reflecting the binding of the hairpin loops, k_{-1} must be $\ll k_{+2}$, as indicated by the linear concentration dependence of k_{obs} monitored by loss of papain activity. Under this steady-state condition, the formation of the initial complex, in which the enzyme already is inactive, is essentially irreversible. Measurements of enzyme activity therefore report only the first step, leading to the observed linear concentration dependence. Thus, k_{-1} must be $\ll 230\ s^{-1}$. A reasonable estimate is $\sim 1\ s^{-1}$, the measured value of k_{diss} for a complex of papain with cystatin A lacking the N-terminal region (Estrada et al., 1999). The condition $k_{-1} \ll k_{+2}$ further means that the first step is not in rapid equilibrium, in contrast to the situation in many other two-step binding reactions (Fersht, 1985). Moreover, for this condition, the parameter $K_{0.5}$ derived from the hyperbolic fits is k_{+2}/k_{+1}, and as k_{ass} is the initial slope of these fits, i.e., $k_{+2}/K_{0.5}$, it follows that $k_{+1} = k_{ass}$. The identical value of k_{ass} obtained in the analyses monitored by loss of papain activity, which reflect only the first step, with those obtained by measurements of probe fluorescence strongly support this conclusion. This internal consistency also attests to the validity of the proposed model. Therefore, k_{+1} should be $\sim 2 \times 10^7\ M^{-1}s^{-1}$. Finally, k_{-2}, the reverse rate constant of the binding of the labeled N-terminal region, can be estimated to $\sim 1 \times 10^{-4}\ s^{-1}$ from the overall K_d and the values for k_{+1}, k_{-1}, and k_{+2} given above. This value for k_{-2}, although dependent on the estimated value for k_{-1}, indicates that the equilibrium of the second step is strongly shifted in favor of binding of the labeled N-terminal region. The proposed mechanism and rate constants are further supported by the value for k_{off} measured by a displacement procedure. For the condition $k_{-1} \ll k_{+2}$, k_{off} is equal to $k_{-2} \cdot k_{-1}/k_2$ and should thus be $\sim 5 \times 10^{-7}\ s^{-1}$, a value that is identical to that measured. It should be noted that the deduced mechanism predicts that lags should be observed in the fluorescence progress curves at high concentrations of the reactant in excess, which was not seen experimentally. However, computer simulations with the DYNAFIT program (Kuzmic, 1996) indicated that such lags most likely would have been obscured by experimental error under the conditions of the analyses, in particular if k_{-1} is higher than the value estimated above.

Previous rapid-kinetics studies monitored by tryptophan fluorescence have given no indication of a two-step binding of unlabeled cystatin A, or other cystatins, to papain (Björk et al., 1989; Lindahl et al., 1992a; Pol et al., 1995). This behavior is in apparent contrast with considerable evidence by X-ray crystallography and NMR that the flexible N-terminal region may bind independent of the two hairpin loops (Bode et al., 1988; Stubbs et al., 1990; Dieckmann et al., 1993; Martin et al., 1995; Ekiel et al., 1997). Our strategy of labeling the N-terminus with a fluorescent reporter group has allowed us to show that the N-terminal region indeed binds independently in a second step with AANS, although not with AEDANS, as the reporter. That this region of unlabeled and AEDANS-labeled cystatin A also binds to the proteinase in a second step after the hairpin loops is supported by several lines of evidence. The similar values of k_{diss} for the complexes of unlabeled

and AANS- or AEDANS-labeled cystatin A with papain thus indicate that the labeling has not affected the interactions of the unlabeled inhibitor with the proteinase. The observed change of probe fluorescence therefore presumably does not reflect a direct interaction of the fluorescent group with the proteinase, but only a transfer of the probe to a less hydrophilic environment at the surface of the enzyme. This conclusion is supported by the finding that deletion of the N-terminal Met residue does not affect the affinity or kinetics of binding of unlabeled cystatin A to papain (Estrada et al., 1999). Moreover, the N-terminal Met is not involved in interactions with papain in the structure of the cystatin B-papain complex (Stubbs et al., 1990), suggesting that a label at a Cys before this Met also would not be in contact with the enzyme. Finally, the alternative explanation that the fluorescence change and two-step reaction seen with AANS–cystatin A reflect a binding or rearrangement of only the probe, after the three binding regions of the inhibitor have interacted with the enzyme in a first step, is highly improbable. In this case, the value for $K_{0.5}$ of $\sim 10 \times 10^{-6}$ M derived from the hyperbolic concentration dependence would reflect the affinity of this initial interaction, a value much lower than that of $\sim 2 \times 10^{-13}$ M measured for the unlabeled inhibitor, in which all three regions interact with the enzyme. Moreover, as the overall K_d for the binding of the labeled inhibitor is $\sim 2 \times 10^{-14}$ M, such a binding or rearrangement would result in a tightening of the interaction of about 10^9-fold, an unlikely large effect.

There are two possible reasons that the binding of the N-terminal region of cystatin A to papain in a second step was not detected for AEDANS-labeled or unlabeled cystatin A in these and previous kinetics studies but was seen for the AANS-labeled inhibitor. The most likely reason is that the binding of the AEDANS-labeled or unlabeled N-terminal region subsequent to the hairpin loops is sufficiently rapid not to be resolved as a separate step in stopped-flow analyses. However, the AANS-labeled region apparently binds more slowly, although in the same manner and with the same interactions as the unlabeled region, thereby allowing its binding to be detected. A higher rate constant for the interaction of the AEDANS-labeled or unlabeled N-terminal region than that of ~ 230 s^{-1} seen for the AANS-labeled inhibitor, of, for example, 1,500 s^{-1}, together with the lower k_{ass} values of $\sim 6 \times 10^6$ or $\sim 3 \times 10^6$ $M^{-1} s^{-1}$ (Table 1), would give a $K_{0.5}$ of $\sim 2.5 \times 10^{-4}$ or $\sim 5 \times 10^{-4}$ M. For these higher $K_{0.5}$ values, an approximately linear increase of k_{obs}, monitored by changes in AEDANS or tryptophan fluorescence, with the concentration of AEDANS-labeled or unlabeled cystatin would have been observed up to the highest concentrations, 40 or 100 μM, respectively, that could be studied in the present and previous studies. It is thus reasonable that the binding of the N-terminal region in a second step would have escaped detection in these studies. Another possible reason that the independent binding of the unlabeled N-terminal region was not seen in past studies is that the tryptophan fluorescence change used to monitor the reaction of unlabeled cystatins with papain may have only reported the binding of the two hairpin loops in the first step. In such a case, the subsequent interaction involving the N-terminal region would not be detected, and simple second-order kinetics would be observed.

The labeling of the N-terminus of cystatin A with the AANS or AEDANS groups was found appreciably to increase k_{ass} for the binding of the inhibitor to papain. The increased rate constant may be caused by an electrostatic steering effect (Schreiber & Fersht, 1996), due to the negative charge of the probe increasing the rate of approach of the labeled inhibitor to the enzyme. Interestingly, as k_{diss} was unaffected or even slightly decreased, the labeling thus transformed cystatin A into a more effective inhibitor of papain.

All mammalian cystatins characterized so far are closely related, and their N-terminal regions, although of different lengths, therefore most likely have similar flexibility as has been demonstrated for cystatin A, cystatin C, and chicken cystatin (Bode et al., 1988; Stubbs et al., 1990; Dieckmann et al., 1993; Martin et al., 1995; Ekiel et al., 1997). Moreover, most target proteinases of these cystatins, among others the lysosomal enzymes, cathepsins K, L, and S, have structures highly similar to that of papain, with easily accessible active sites (Fujishima et al., 1997; McGrath et al., 1997; Zhao et al., 1997; McGrath et al., 1998; Guncar et al., 1999). Independent binding of the N-terminal region of cystatins in a second step after the two hairpin loops in the manner proposed in this work therefore presumably is a general feature of cystatin inhibition of such enzymes.

Materials and methods

Construction of the +Cys–cystatin A vector

A DNA fragment introducing the codon for a Cys residue before that for Met-1 in the cDNA of human cystatin A was amplified by PCR. The template was the previously described expression vector for cystatin A (Estrada et al., 1998), which harbors the cDNA for cystatin A, a removable His-tag and the signal sequence for outer membrane protein A that targets the expressed protein to the periplasmic space of *Escherichia coli*. The upstream primer was 5′-GCTC AGGCGACCATGGGCCATCATCATC and included a restriction site for *Nco* I (underlined). The downstream primer was 5′-GCC CCCGGGTATCAT*ACA*CTTGTCGTCGTCGTCGATATGG, and included an *Xma* I site (underlined) as well as the codon for the additional Cys (in italics). The original vector was digested with *Nco* I and *Xma* I, which excised a fragment of 74 bp including the bases coding for the His-tag, the enterokinase recognition sequence and the first three residues of cystatin A. The remainder of the vector was purified, and the excised fragment was replaced by ligation with the PCR product (77 bp), which had been cleaved with the same restriction enzymes. The resulting plasmid was transformed into the *E. coli* strain MC 1061 and propagated on agar plates supplemented with 100 μg/mL ampicillin. Surviving colonies were picked, and the DNA sequence of the replaced region of the expression vector was determined.

The +Cys–cystatin A variant was expressed in *E. coli* and purified by Ni^{++} affinity chromatography as described earlier (Estrada et al., 1998), except that the washing of the column with 75 mM imidazole was omitted. After addition of DTT to 5 mM, the sample was dialysed against 70 mM Tris-HCl, pH 7.4, containing 2 mM $CaCl_2$ and 0.5 mM DTT, and was then digested with enterokinase (Biozyme, Blaenavon, UK) at an enzyme to fusion protein weight ratio of 1 to 500 for 18 h at 37 °C. The enterokinase was inactivated by addition of N-tosyl-L-phenylalanine chloromethyl ketone to 10 μM, and the cleaved cystatin A variant was recovered by reapplying the sample to the Ni^{++} column and collecting the unbound fraction.

Labeling of the N-terminus

+Cys–cystatin A (2–7 mg) was incubated for 5 min in 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, and 5 mM DTT, pH 7.4. The

sample was then applied to a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with the same Tris buffer, although at pH 8.2 and containing only 0.5 mM DTT. The effluent protein was collected in a tube containing IAANS or IAEDANS (Molecular Probes, Inc., Eugene, Oregon) in a 10-fold or fourfold molar ratio to the protein, respectively (in excess of the amount expected to react with the DTT in the buffer). Labeling was done at room temperature for 30 min or 6 h with IAANS or IAEDANS, respectively. Unreacted labeling reagent and its reaction product with DTT were then removed on a PD-10 column equilibrated with 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mM EDTA, pH 7.4, followed by dialysis of the sample against the same buffer.

Protein concentrations

The purification and activation of papain (EC 3.4.22.2) have been reported elsewhere (Lindahl et al., 1988; Björk et al., 1994). Concentrations of the enzyme were determined by absorption measurements at 280 nm from a molar absorption coefficient of 55,900 $M^{-1}\,cm^{-1}$ (Lindahl et al., 1988). The molar absorption coefficient at 280 nm for cystatin A, 8,800 $M^{-1}\,cm^{-1}$ (Pol et al., 1995), was used also for the +Cys–cystatin A variant and the labeled cystatin A forms. In calculations of concentrations of the latter, the contribution of the labeling group to the observed absorbance at 280 nm was first subtracted. This contribution was obtained from absorption spectra of the reaction products between the labeling reagents and an excess of 2-mercaptoethanol. The concentration of AANS-cystatin A was also measured by quantitative amino acid analysis (Pol et al., 1995). Concentrations of the AANS and AEDANS groups were determined from the absorbances at 327 and 336 nm, respectively, with the use of molar absorption coefficients of 26,000 and 5,700 $M^{-1}\,cm^{-1}$, respectively (Haugland, 1996).

Fluorescence

Fluorescence emission spectra were measured in an F-4000 spectrofluorimeter (Hitachi, Tokyo, Japan), essentially as described earlier (Lindahl et al., 1988). Excitation was at 327 nm with excitation and emission bandwidths of 10 nm for AANS–cystatin A and at 335 nm with bandwidths of 5 nm for AEDANS–cystatin A.

Stoichiometry of papain binding

The stoichiometries of binding of the labeled cystatin A forms to papain were measured by titrations, monitored by increase of the fluorescence of the N-terminal probe accompanying the binding, of 1 μM labeled inhibitor with the enzyme. The excitation wavelengths were 327 and 335 nm, and the emission wavelengths 450 and 490 nm for AANS and AEDANS–cystatin A, respectively. The binding stoichiometries were evaluated by nonlinear least-squares regression (Lindahl et al., 1988, 1992a). The stoichiometry of binding of AANS–cystatin A to papain was also obtained by titrations in which the residual activities of a series of enzyme samples at a constant concentration (20 nM) were measured with a fluorogenic substrate after addition of increasing concentrations of the inhibitor (Estrada et al., 1998).

Association kinetics

The kinetics of association of AANS– or AEDANS–cystatin A with papain were analyzed under pseudo-first-order conditions by stopped-flow measurements in an SX-17MV instrument (Applied Biophysics, Leatherhead, UK), essentially as in earlier work (Björk et al., 1994, 1995; Turk et al., 1995; Estrada et al., 1998). The reactions were monitored either by product formation resulting from papain cleavage of a fluorogenic substrate present in the reaction mixture, or by the increase of probe fluorescence accompanying the binding. In the experiments monitored by substrate cleavage, the concentration of AANS– or AEDANS–cystatin A was 10-fold over that of the enzyme and varied from 1 to 8 μM. The concentration of the substrate, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan), was 10 μM, and substrate consumption was $<5\%$. Product formation was monitored with an excitation wavelength of 370 nm and with an emission filter with 50% transmission at 400 nm. In the analyses monitored by the increase of probe fluorescence, two sets of experiments were performed with AANS–cystatin A. In the first of these, the concentration of papain was 10-fold higher than that of the labeled inhibitor and was varied from 1 to 15 μM. In the second set, the molar ratio was reversed, and the concentration of the labeled inhibitor was varied from 1 to 8 μM. In the corresponding analyses with AEDANS–cystatin A, only one set of experiments were done, in which the molar ratio of papain to labeled inhibitor was 10-fold, and the enzyme concentration was varied from 5 to 40 μM. The excitation wavelengths were 327 and 335 nm for AANS– and AEDANS–cystatin A, respectively, and the emission was recorded through the filter with $\sim$50% transmission at 400 nm. Observed pseudo-first-order rate constants (k_{obs}) were calculated by nonlinear least-squares regression analyses of the progress curves (Björk et al., 1994, 1995). k_{obs} values determined in the presence of substrate were corrected for substrate competition (Björk et al., 1994, 1995) with a K_m value of 60 μM (Hall et al., 1992).

Dissociation kinetics

The kinetics of dissociation of the complexes between AANS– or AEDANS–cystatin A and papain were analyzed by essentially irreversibly trapping the enzyme dissociated from the complex with a large excess of chicken cystatin (Björk et al., 1989; Lindahl et al., 1992a; Pol et al., 1995). Chicken cystatin binds much tighter to papain than cystatin A (Pol et al., 1995), thereby preventing reassociation of the liberated enzyme with cystatin A. In the experiments with AANS–cystatin A, the concentrations of the complex with papain were 0.7–1 μM, and those of the displacing chicken cystatin (form 1) were 20–50 μM. The reactions were monitored by the decrease of AANS fluorescence accompanying the dissociation, measured with excitation and emission wavelengths of 327 and 450 nm, respectively. In the analyses with AEDANS–cystatin A, the complex concentration was 10 μM, and that of chicken cystatin (form 2) was 100 μM. Because of the low fluorescence decrease resulting from the dissociation, the rate was instead monitored by the appearance of the tight complex between the displacing chicken cystatin and the liberated papain by chromatography on a Mono-Q (Amersham Pharmacia Biotech) ion exchange column (Lindahl et al., 1992a). The dissociation rate constants were obtained by nonlinear least-squares regression analysis of the data to the equation for a single exponential reaction (Björk et al., 1989).

Experimental conditions

All analyses of the interactions between the labeled inhibitors and papain were carried out at 25.0 $\pm$ 0.2 °C in 50 mM Tris-HCl,

100 mM NaCl, 0.1 mM EDTA, pH 7.4. The buffer contained 1 mM DTT in the stopped-flow measurements and also 0.01% (w/v) Brij 35 in the experiments in the presence of substrate.

Miscellaneous procedures

SDS-PAGE under reducing conditions was run on 15% gels (Laemmli, 1970). N-terminal sequence analysis and quantitative amino acid analysis were done as in Pol et al. (1995).

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